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Differential Susceptibility of Guanine Nucleotide-binding Proteins to Pertussis Toxin-catalyzed ADP-ribosylation in Brain Membranes of Two Congeneric Marine Fishes

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Abstract. Pertussis toxin-catalyzed [^{32}P]ADP-ribosylation was used to probe the guanine nucleotide binding regulatory proteins G_i and G_o in brain membranes from two scorpaenid fishes, *Sebastolobus alascanus* and *S. altivelis*. The membranes of the two species exhibit a differential sensitivity to [^{32}P]ADP-ribosylation produced by a fixed concentration of pertussis toxin. The membranes from the deeper-living *S. altivelis* consistently incorporated more [^{32}P]ADP than the membranes from *S. alascanus*. Proteins of 39 and 41 kDa are specifically labeled in both species, corresponding to the apparent molecular masses of the α subunits of G_i and G_o. At 5°C the ribosylation reaction is linear for at least 7 h. The pertussis toxin concentration-response relationship was evaluated with concentrations of pertussis toxin from 0 to 100 ng/μl. The extent of [^{32}P]ADP-ribosylation was quantified by autoradiography and computer-assisted image analysis. The EC_{50} values for pertussis toxin were similar for the two species, but the maximum level of [^{32}P]ADP-ribosylation was significantly greater in *S. altivelis* brain membranes. Because the heterotrimeric holoprotein is the substrate for ribosylation, the modulatory effects of the guanyl nucleotides GDP and GTPγS on the ribosylation were assessed. GDP increased [^{32}P]ADP-ribosylation of the α subunits in *S. altivelis*. Only the highest concentration tested (1000 μM) increased [^{32}P]ADP-ribosylation in *S. alascanus* brain membranes and only to a modest extent. Increasing concentrations of GTPγS suppressed [^{32}P]ADP-ribosylation in *S. alascanus* brain membranes, presumably by promoting dissociation of the holotrimer. GTPγS had much less of an effect on the *S. altivelis* brain membranes. These differences in the extent of ADP-ribosylation and the modulatory effects of guanyl nucleotides may reflect different coupling efficiencies of G proteins and receptors. The expression of the α and β subunits of G_i and G_o in the two *Sebastolobus* species, the deep-sea morid teleost fish *Antimora rostrata*, and the rat were compared by Western immunoblotting of brain membranes with antipeptide antisera. Levels of $G_{i\alpha 3}$ were 63% higher in brain membranes of *S. altivelis* than those in *S. alascanus*. The levels of $G_{i\alpha 1}$, $G_{i\alpha 2}$, G_o and β_{36} were similar in the two species. Although the complement of G proteins identified by the array of antisera used was similar in all the species, there appears to be additional diversity of α subunits in the teleost brain membranes. In fish, antiserum to $G_{o\alpha}$ reacted with an additional 41 to 42 kDa protein that was not expressed in rat brain.

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Introduction

The hydrostatic pressures characteristic of the deep sea exert profound effects on the physiology and biochemistry of organisms in this extensive habitat (Siebenaller, 1987, 1991; Siebenaller and Somero, 1989). Among the processes affected is guanine nucleotide binding protein (G protein)-coupled transmembrane signaling (Siebenaller *et al.*, 1991; Siebenaller and Murray, 1993). In A_1 adenosine receptor-modulation of adenylyl cyclase in teleost brain membranes, pressure affects agonist efficacy (Siebenaller *et al.*, 1991; Siebenaller and Murray, 1993), the coupling of the receptor to adenylyl cyclase (Siebenaller *et al.*, 1991), and the enzymatic activities of components of the system,

i.e., adenylyl cyclase activity (Siebenaller *et al.*, 1991) and the high-affinity GTPase activity of α subunits of G proteins (Siebenaller and Murray, 1993).

G proteins couple a diverse superfamily of cell surface receptor proteins (estimated at up to 1000, *e.g.*, Barinaga, 1991) to a variety of effector elements such as adenylyl cyclase, ion channels, and phospholipases (Birnbaumer *et al.*, 1990). Because G proteins are central to transmembrane signaling, physical factors, such as pressure, may be critical as selective forces influencing adaptation and shaping the functional characteristics of G proteins.

G proteins are heterotrimers and the subunits are designated α, β, and γ. The classes of G proteins are defined by the α subunit type (Gilman, 1987), and further diversity of the α subunits has been documented by molecular genetic techniques (*e.g.*, Simon *et al.*, 1991). The general features of the model of G protein-coupled signaling are briefly described here (see Gilman, 1987, and Birnbaumer *et al.*, 1990). Agonist-liganded receptors interact with G proteins, promoting the binding of GTP to the guanine nucleotide binding site on the α subunit. GTP binding evokes a conformational change (Yi *et al.*, 1991), causing the protein to dissociate into $\alpha \cdot$GTP and a $\beta\gamma$ dimer (Gilman, 1987; Birnbaumer *et al.*, 1990; Boege *et al.*, 1991). The activated $\alpha \cdot$GTP complex and the $\beta\gamma$ dimer interact with the target enzyme (Gilman, 1987; Lefkowitz, 1992; Birnbaumer, 1992). Signaling is terminated by the hydrolysis of bound GTP to GDP by the intrinsic GTPase activity of the α subunit and the subsequent reassociation of the α and $\beta\gamma$ subunits.

The inhibitory G protein (G_i) plays a role in receptor-mediated inhibition of adenylyl cyclase, whereas G_o is a common G protein in brain membranes. These two classes of G proteins are specific substrates for mono-ADP-ribosylation by pertussis toxin (Katada and Ui, 1982; Gierschik, 1992). Pertussis toxin catalyzes the transfer of an ADP-ribose moiety from NAD^+ to a specific cysteine four residues from the carboxyterminus of the α subunit in the heterotrimer. ADP-ribosylation prevents the coupling of the modified G protein to its receptor and therefore a response to agonists (Gierschik, 1992). Because the heterotrimeric holoprotein is the substrate for ADP-ribosylation (Neer *et al.*, 1984; Van Dop *et al.*, 1984), pertussis toxin has been used to characterize the subunit aggregation state and the conformation of G proteins (*e.g.*, Yi *et al.*, 1991), as well as the interaction of receptors and G proteins (*e.g.*, van der Ploeg *et al.*, 1992).

The α subunits of G_i and G_o serve as transduction elements in A_1 adenosine receptor transmembrane signaling (Linden, 1991). We have used pertussis toxin-catalyzed ADP-ribosylation to probe the G_i and G_o proteins of two *Sebastolobus* species in order to identify species differences that might be correlated with the differences in the pressure sensitivity of A_1 adenosine receptor-mediated inhibition of adenylyl cyclase (Siebenaller *et al.*, 1991). These two scorpaenid fishes of the genus *Sebastolobus* have similar life histories, experience similar temperatures, but occur at different depths (Hubbs, 1926; Siebenaller and Somero, 1978). With these species, fine-scale adaptations to pressure have been delineated without the potentially confounding effects of other environmental variables or phylogenetic distance (*e.g.*, Siebenaller, 1984a, b, 1987).

We observed previously that the extent of [^{32}P]ADP-ribosylation produced by a fixed concentration of pertussis toxin is different in the two *Sebastolobus* species (Siebenaller and Murray, 1990; Siebenaller *et al.*, 1991). Brain membranes from the deeper-living *S. altivelis* incorporate more [^{32}P]ADP than *S. alascanus* membranes (Siebenaller *et al.*, 1991). Our previous work (*e.g.*, Murray and Siebenaller, 1987; Siebenaller and Murray, 1990; Siebenaller *et al.*, 1991) suggested that the coupling of G proteins to receptors would be different in species adapted to different pressure regimes; *e.g.*, the two *Sebastolobus* species that experience different pressure regimes yet have brain membranes with identical phospholipid and fatty acid compositions (Siebenaller *et al.*, 1991). A less tightly coupled and more flexible signaling complex may be required for optimal function in the more highly ordered membranes which result at the higher pressures experienced by the deeper-living *S. altivelis* (Siebenaller *et al.*, 1991; see also Casadó *et al.*, 1992; Shinitzky, 1984).

In the present study we have confirmed the difference in pertussis toxin-catalyzed incorporation of [^{32}P]ADP into the α subunits of G_i and G_o of the two *Sebastolobus* species. We have tested two possible reasons for this difference in [^{32}P]ADP ribosylation: that the G-proteins of the two species are differently suitable as substrates for ribosylation (*i.e.*, that the ribosylation site of *S. altivelis* is more susceptible to pertussis toxin); or that the levels of the G protein substrates in the two species are different. We have quantified the steady state levels and subtypes of G_i and G_o α and β subunits by Western immunoblot analyses as a measure of the differential expression of subunits in the two species. To examine and characterize the coupling of G proteins to receptors in the two species, we have tested the effects of guanyl nucleotides on pertussis toxin-catalyzed ribosylation. We relate these data to differences in the coupling of the A_1 adenosine receptor to adenylyl cyclase in brain membranes of the two *Sebastolobus* species.

Materials and Methods

Specimens

Demersal adults of *Sebastolobus* (Scorpaenidae) were collected by otter trawl off the coast of Oregon at their typical depths of abundance on two cruises of the R/V *Wecoma*. *S. alascanus* adults are common between 180

and 330 m; the adults of *S. altivelis* are found between 550 and 1300 m (Miller and Lea, 1976). Demersal *Antimora rostrata* (Moridae) were collected at their typical depths of abundance, 850–2500 m (Haedrich and Merret, 1988), off the coast of Newfoundland, Canada, on a cruise of the R/V *Gyre*. Brain tissue was dissected, frozen in liquid nitrogen at sea, and transported to the laboratory where tissues were maintained at −80°C until used.

Frozen rat brains were obtained from Pel-Freez (Rogers, Arkansas) and forebrains were dissected following thawing of the whole brain.

Preparation of brain membranes

Membranes were homogenized with a Dounce (Pestle A) in 40 volumes of 50 m*M* Tris-HCl, pH 7.6 at 5°C, containing 0.3 mg/ml soybean trypsin inhibitor and 3.4 mg/ml bacitracin. The homogenate was centrifuged at 27,000 × *g* (0 to 4°C) for 10 min. The pellet was resuspended in 40 volumes of buffer and recentrifuged. The pellet was resuspended in 40 volumes of buffer and used in the ribosylation assays.

The protein concentration was determined prior to experimentation to permit adjustment of the samples to equivalent protein concentrations. Protein was determined by the method of Lowry *et al.* (1951) following solubilization of the samples in 0.5 *M* NaOH. Bovine serum albumin (Sigma) was used as the standard.

[^{32}P]ADP ribosylation

Pertussis toxin-catalyzed [^{32}P]ADP ribosylation of α subunits of G_i and G_o followed the procedures described in Siebenaller and Murray (1990). Pertussis toxin was activated in 100 m*M* Tris-HCl, pH 8.0 with 50 m*M* dithiothreitol for 1 h at room temperature. The 100 μl incubation mixture routinely contained 100 m*M* Tris-HCl, pH 7.6 at the incubation temperature of 5°C, 25 m*M* dithiothreitol, 2 m*M* ATP, 2 μCi NAD, 1.5 μg soybean trypsin inhibitor, 15 μg bacitracin, 2 μg pertussis toxin, and 10 to 20 μg of membrane protein. The incubation was stopped by adding 50 μl of stop solution (3% sodium dodecyl sulfate, 42% glycerol, 15% 2-mercaptoethanol, 200 m*M* Tris-HCl, pH 6.8 at 20°C) and the mixture was boiled for 5 min. The denatured samples were subjected to sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) in 1.5 mm thick 12.5% acrylamide gels following Laemmli (1970). The gel was stained with 0.25% Serva Blue R (Serva Fine Biochemicals, Westbury, New York) in 25% 2-propanol, 10% acetic acid, destained, and dried. The dried gels were apposed to Kodak (Rochester, New York) X-Omat AR film. DuPont Cronex Lightning Plus intensifying screens were used. The developed autoradiograms were digitized and quantified with an MCID system (Imaging Research, Inc., St. Catherine, Ontario, Canada).

Immunological quantification of G proteins

Membranes were diluted to the appropriate protein concentration and electrophoresed in Laemmli sodium dodecyl sulfate mini-gels (0.75 mm thick, 4.5% acrylamide stacking/12.5% acrylamide resolving) in a BioRad MiniProtean II electrophoresis unit. When using AS/7 antiserum to detect $G_{i\alpha 1}$ and $G_{i\alpha 2}$, we supplemented the resolving gel with 4 *M* urea to enhance separation of these proteins. For routine assays, 10 μg samples were diluted in 2× Laemmli sample buffer for a final volume of 15 μl and heated to 95°C for 3 min. Samples of each species were run on each gel.

Proteins were transferred from the gels to nitrocellulose membranes (Schleicher & Schuell, BA85, 0.45 μm) on a Sartoblot II-S transfer block (Sartorius). Transfer was accomplished in 12 h at 100 mA (constant current) using 25 m*M* Tris, 150 m*M* glycine, 10% methanol, pH 8.3. The nitrocellulose membrane was stained in 0.2% Ponceau S (Sigma Chemical Co.) and the polyacrylamide gels were stained with Coomassie Brilliant Blue R (Sigma) to assess completeness of transfer. After destaining in deionized water, the nitrocellulose was blocked for 1 h at room temperature with 1% nonfat dried milk (Carnation) in Tris-buffered saline (TBS: 20 m*M* Tris HCl, 150 m*M* NaCl, pH 7.5 and 0.05% Tween-20). The washed nitrocellulose membrane was incubated overnight at 4°C with primary rabbit antisera diluted 1:1000 in 1% dried milk/TBS. Following multiple washes in TBS and 1% dried milk/TBS, the blots were incubated for 2 h at room temperature with goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) diluted 1:1000 in 1% dried milk/TBS. The nitrocellulose was again washed in TBS, and then washed in alkaline phosphatase buffer (0.1 *M* Tris-HCl, 0.1 *M* NaCl, 2 m*M* $MgCl_2$, 1 μ*M* $ZnCl_2$, 25 m*M* diethanolamine, pH 9.55). Enzyme activity was assayed using 3 mg/ml nitroblue tetrazolium chloride and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt (Sigma) in alkaline phosphatase buffer. Color development was stopped after 5 to 10 min by washing with deionized water. The blots were digitized and quantified using the MCID system.

Data analysis

Statistical comparisons were made by Student's *t*-test and analysis of variance using Instat (GraphPad, San Diego, California).

Reagents

[Adenylate-^{32}P]-nicotinamide adenine dinucleotide ([^{32}P]NAD, 31.31 Ci/mmol) was from DuPont NEN

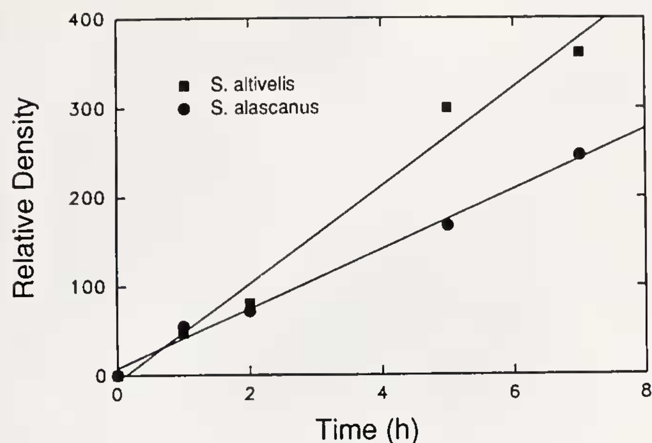

Figure 1. Time course of pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of *Sebastolobus altivelis* (squares) and *S. alascanus* (circles) brain membranes at 5°C. Preparations were incubated with 2 μCi [^{32}P]NAD and 2 μg preactivated pertussis toxin. The samples were denatured and subjected to SDS-PAGE. The dried gels were exposed to x-ray film. The relative optical density was determined by densitometric analysis of the autoradiogram. The results are from a single experiment which was replicated twice with similar results.

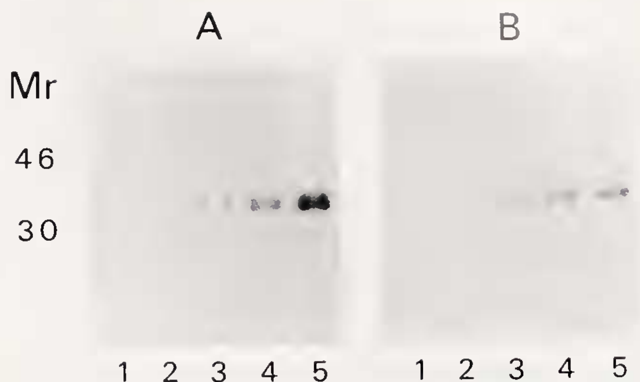

Figure 2. Autoradiogram of pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of *Sebastolobus altivelis* (A) and *S. alascanus* (B) brain membranes. Membranes were incubated for 6 h at 5°C with 2 μCi [^{32}P]NAD in the presence of increasing concentrations of pertussis toxin. The concentrations of pertussis toxin used are Lane 1, 5 ng/μl; Lane 2, 10 ng/μl; Lane 3, 25 ng/μl; Lane 4, 50 ng/μl, and Lane 5, 100 ng/μl. The membranes were subjected to SDS-PAGE and labeled proteins were detected by autoradiography. The results are from a single representative experiment that was repeated three times.

(Boston, Massachusetts). Pertussis toxin (islet activating protein) was from List Biological Laboratories, Inc. (Campbell, California). Water was processed through a four-bowl Milli-Q purification system (Millipore, Bedford, Massachusetts). Molecular weight standards were from BioRad. The antipeptide antisera obtained from DuPont NEN were AS/7 (recognizes $G_{i\alpha 1}$ and $G_{i\alpha 2}$, Goldsmith *et al.*, 1987, 1988), EC/2 (recognizes $G_{i\alpha 3}$ and $G_{o\alpha}$, Simonds *et al.*, 1989) GC/2 (recognizes $G_{o\alpha}$, Spiegel, 1990) and MS/1 (recognizes G_{β}, Goldsmith *et al.*, 1988). These antisera had been raised to deduced consensus sequence peptides of the carboxy- and amino-termini of G protein subunits of mammalian species (*e.g.*, McKenzie *et al.*, 1988; Mumby and Gilman, 1991). Other reagents were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Results

The time course of pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of brain membranes from *S. altivelis* and *S. alascanus* is depicted in Figure 1. At 5°C, the labeling of G protein α subunits was linear for 7 h in both species.

The pertussis toxin concentration-response relationships in both species were evaluated in a fixed 6-h incubation. Equivalent amounts of *S. altivelis* and *S. alascanus* membrane protein were loaded on SDS-polyacrylamide gels following the [^{32}P]ADP-ribosylation reaction. The dependence of [^{32}P]ADP-ribosylation on pertussis toxin concentration is shown in the autoradiogram in Figure 2. In brain membranes from both species there was no specific labeling in the absence of pertussis toxin. The extent of [^{32}P]ADP-ribosylation increased as a function of pertussis toxin concentration (5–100 ng/μl) in both species. The labeling was consistently greater in *S. altivelis* membranes. Proteins of 39 kDa and 41 kDa were radiolabeled in both species. Image analysis of the autoradiograms per-

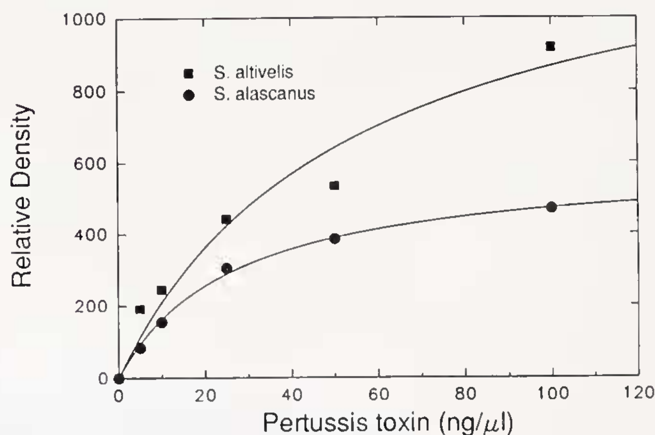

Figure 3. Concentration-response relationship for pertussis toxin-catalyzed [^{32}P]ADP-ribosylation of *Sebastolobus altivelis* (squares) and *S. alascanus* (circles) brain membranes for 6 h at 5°C. The curves are the best fits to the data using the logistic function: $E = (E_{max})/(1 + EC_{50}/[PTX])$. E is the effect in units of relative optical density and E_{max} is the maximum effect. EC_{50} is the concentration of pertussis toxin (PTX) which produced 50% of the maximum effect, and [PTX] is the concentration of pertussis toxin. The maximum extent of [^{32}P]ADP-ribosylation in *S. altivelis* brain membranes was 2.2-fold greater than the level of [^{32}P]ADP-ribosylation in *S. alascanus* membranes. The results depicted represent the fits to pooled data from three separate experiments. The parameter estimates derived from this analysis are shown in Table I.

Table I

Analysis of pertussis toxin concentration-response relationships in Sebastolobus altivelis *and* S. alascanus *brain membranes*

Species	Pertussis toxin EC_{50} (ng)	E_{max} (ROD)
S. alascanus	26.7 ± 3	597 ± 25
S. altivelis	53.5 ± 27	1329 ± 325

E_{max} units are relative optical density (ROD).

Table II

Quantitation of material in brain membranes of Sebastolobus alascanus *and* S. altivelis *immunoreactive to antibodies directed against α and β subunits of G_i and G_o*

Subunit	*S. alascanus*	*S. altivelis*
$G_i\alpha_1$	1 (9)	1.14 ± 0.29 (8)
$G_i\alpha_2$	1 (10)	1.09 ± 0.19 (9)
$G_i\alpha_3$	1 (12)	1.63 ± 0.40* (12)
$G_o\alpha$	1 (13)	1.12 ± 0.28 (13)
β_{36}	1 (14)	1.06 ± 0.10 (11)

The data are standardized to *S. alascanus*. The number of determinations is given in parentheses.

* Value significantly different from the 1.00 value at $P < 0.05$.

mitted quantification of the relative optical density of each lane. The resultant pertussis toxin concentration-response curves are shown in Figure 3. The maximum level of [^{32}P]ADP-ribosylation is significantly greater in *S. altivelis* brain membranes than in *S. alascanus* membranes (Fig. 3, Table I). The EC_{50} values for pertussis toxin in the two species were not significantly different (Table I).

The increase in the extent of pertussis toxin-induced labeling of G protein α subunits in *S. altivelis* could be due to a greater accessibility of the ribosylation sites of the $G_{i\alpha}$ and $G_{o\alpha}$ substrates, or to elevated quantities of $G_{i\alpha}$ and $G_{o\alpha}$ or $G_{\beta\gamma}$. Western immunoblots were employed to quantify the levels of G protein α and β subunits in brain membranes of the two *Sebastolobus* species. For these studies, another deep-living species, *Antimora rostrata*, exemplified the pattern and diversity of immunoreactive material in brain membranes from another marine fish family (Moridae). Rat (*Rattus rattus*) brain membranes were employed as a reference to aid in identifying the immunoreactive material. Equal amounts of membrane protein from each species were loaded on gels and an array of antipeptide antisera were employed to detect $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, $G_{o\alpha}$ and the β subunit. Standard curves were generated for each antiserum by varying the amount of membrane protein loaded on gels. The intensity of staining of G protein subunits was within the linear range of immunoreactivity for the results shown. Representative immunoblots for $G_{i\alpha1}$, $G_{i\alpha2}$, $G_{i\alpha3}$, and $G_{o\alpha}$ are depicted in Figure 4.

Using antiserum EC/2 to detect $G_{i\alpha3}$, we observed a single band in brain membranes of the four species. This band from brain membranes of *A. rostrata* and *R. rattus* migrated as an approximately 41 kDa protein. The corresponding protein in both *Sebastolobus* species had a lesser apparent molecular mass. The intensity of staining of this protein was consistently greater (mean = 1.63-fold) in *S. altivelis* than in *S. alascanus* brain membranes (Table II). Antiserum EC/2 was raised against the carboxyterminal decapeptide of mammalian transducin-α (Goldsmith *et al.*, 1987) and crossreacts somewhat with both $G_{o\alpha}$ and $G_{i\alpha1}$ (Simonds *et al.*, 1989). Because only a single band was identified in each species, these bands are tentatively identified as $G_{i\alpha3}$.

Antiserum GC/2 was used to detect $G_{o\alpha}$. As shown in Figure 4, this antiserum recognized a single band of ap-

Figure 4. Immunoblots of *Sebastolobus altivelis* (Ve), *S. alascanus* (Sc), *Antimora rostrata* (Ro), and *Rattus rattus* (Rat) brain membranes obtained with peptide antisera specific for G protein α subunits. Brain membranes were subjected to SDS-PAGE, and the separated proteins were transferred to nitrocellulose membranes. Immunoblotting was performed with antiserum EC/2, selective for $G_{i\alpha3}$, antiserum GC/2, selective for $G_{o\alpha}$ and antiserum AS/7 which recognizes $G_{i\alpha1}$ and $G_{i\alpha2}$. The positions of molecular weight standards are indicated.

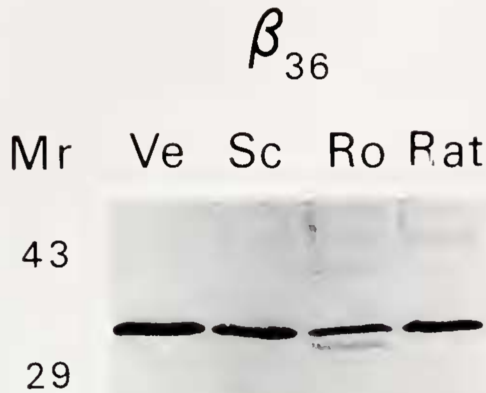

Figure 5. Immunoblots of *Sebastolobus altivelis* (Ve), *S. alascanus* (Sc), *Antimora rostrata* (Ro), and *Rattus rattus* (Rat) brain membranes obtained using a peptide antiserum (MS/1) which recognizes the β subunit (36 kDa) of G proteins.

proximately 39 kDa in rat brain membranes, but two immunoreactive proteins were detected in all three marine fishes. In addition to a heavily stained band of approximately 39 kDa, which likely represents $G_{o\alpha}$, brain membranes from the marine teleosts displayed immunoreactive proteins of apparent molecular masses of 41 to 42 kDa. There were no significant differences between the two *Sebastolobus* species in the intensity of immunoreactive bands detected with the antiserum GC/2 (Table II).

The antipeptide antiserum AS/7 was used to identify $G_{i\alpha 1}$ and $G_{i\alpha 2}$. These α subunits in rat brain were readily resolved in the presence of 4 *M* urea in the running gel (Fig. 4). This doublet was not resolved as well in the three marine fishes, where the band tentatively identified as $G_{i\alpha 2}$ migrated somewhat slower than the corresponding protein in rat brain membranes. There were no significant differences in the levels of $G_{i\alpha 1}$ or $G_{i\alpha 2}$ between the *Sebastolobus* species (Table II).

Immunoblots of G protein β subunits are depicted in Figure 5. The antiserum MS/1 was used to quantify the 36 kDa β subunits, and the intensity of these bands did not differ significantly between the *Sebastolobus* species (Table II).

The substrate for the pertussis toxin-catalyzed ADP-ribosylation reaction is the heterotrimeric holoprotein (Neer *et al.*, 1984; Van Dop *et al.*, 1984), and as a consequence, guanyl nucleotides are capable of modulating the sensitivity of G proteins to pertussis toxin (*e.g.*, Gierschik, 1992). We therefore compared the guanyl nucleotide regulation of ADP-ribosylation in *S. altivelis* and *S. alascanus*. The aim was to examine the differential susceptibility of the G protein α subunits to modification by pertussis toxin.

Increasing concentrations of guanosine 5'-diphosphate (GDP) included in the incubation with pertussis toxin augmented the extent of [^{32}P]ADP-ribosylation in *S. altivelis* brain membranes (Fig. 6). Only the highest concentration tested, 1000 μM GDP, had an effect in *S. alascanus* brain membranes, and this effect was modest (Fig. 6).

Incubation with the nonhydrolyzable GTP analog guanosine 5'-O-(3-thiotriphosphate) (GTPγS) effected a marked suppression of [^{32}P]ADP-ribosylation in *S. alascanus* brain membranes (Fig. 7). [^{32}P]ADP-ribosylation of the G protein α subunits of *S. altivelis* membranes was relatively insensitive to the effect of GTPγS (Fig. 7). The concentration-dependent inhibition of pertussis toxin-catalyzed ADP-ribosylation produced by GTPγS in *S. alascanus* brain membranes presumably reflects a dissociation of the G_i and G_o heterotrimers.

Discussion

Pertussis toxin catalyzes the incorporation of significantly more [^{32}P]ADP into brain membranes of *S. altivelis* than of *S. alascanus* (Siebenaller *et al.*, 1991; Fig. 2 and 3, Table I). Although *S. altivelis* brain membranes have

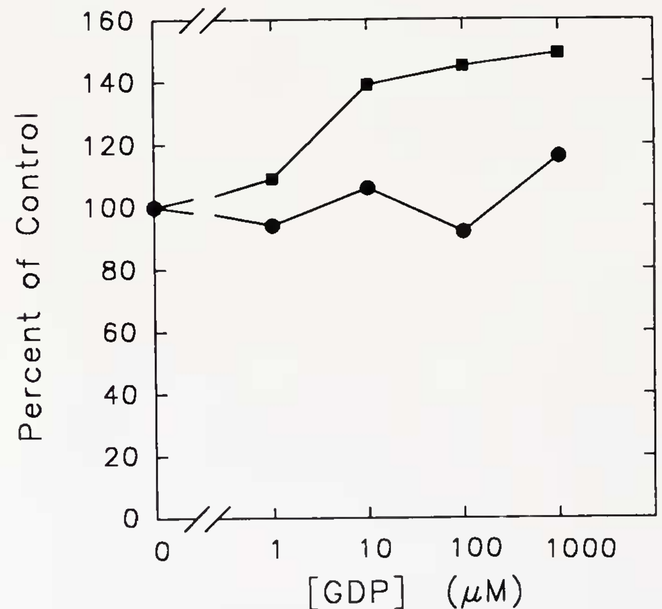

Figure 6. Effects of GDP on pertussis toxin-catalyzed [^{32}P]ADP ribosylation of G protein α subunits in brain membranes of *S. altivelis* (squares) and *S. alascanus* (circles). Membranes were incubated with 2 μCi [^{32}P]NAD and 2 μg preactivated pertussis toxin in the presence of increasing concentrations of GDP. Incubations were at 5°C for 6 h. SDS-PAGE and autoradiography were carried out as described in Materials and Methods. The ordinate values represent the relative optical density of autoradiograms expressed as % of control value determined in the absence of GDP. The results depicted are derived from a single experiment which was replicated twice with similar results.

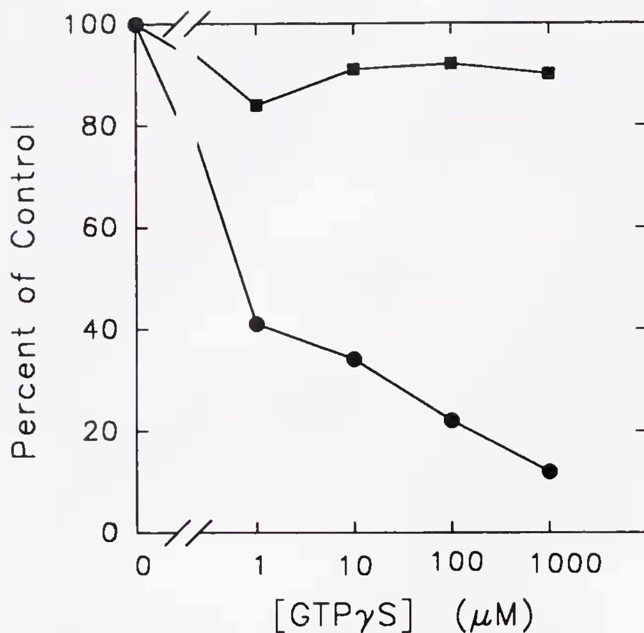

Figure 7. Effects of GTPγS on pertussis toxin-catalyzed [32P]ADP-ribosylation of G protein α subunits in brain membranes of *S. altivelis* (squares) and *S. alascanus* (circles). The methods and data presentation are as described in the legend to Figure 6.

a 63% higher level of one subtype of pertussis toxin substrate, $G_{i\alpha 3}$ (Table II), a simple increase in the amount of G protein substrates cannot adequately explain the differences between the two species. There are also qualitative differences in the pertussis toxin-catalyzed reaction; *i.e.*, the guanyl nucleotides GDP and GTPγS modulate the [32P]ADP-ribosylation reaction differently in the membranes of the two species (Figs. 6, 7). These experiments indicate that *S. alascanus* brain membranes contain lower quantities of one type of pertussis toxin substrate, and that the ribosylation sites of the substrates are less accessible to pertussis toxin.

The differences between the two species in the guanyl nucleotide modulation of ribosylation may relate to the interactions of G_i and G_o with receptors. The A_1 adenosine receptors of *S. alascanus* brain membranes are more tightly coupled to G proteins, and a larger fraction exist coupled, than are those of *S. altivelis* (Murray and Siebenaller, 1987). This conclusion is based on the greater agonist affinity of the A_1 adenosine receptor and the larger fraction of receptors in the high affinity state in *S. alascanus* (Murray and Siebenaller, 1987). The present study suggests that this relatively tighter coupling may be characteristic of other receptors. Tighter receptor-G protein interactions might explain the reduced susceptibility to pertussis toxin-catalyzed ADP-ribosylation in *S. alascanus* membranes; *i.e.*, it would reduce accessibility of the carboxyterminal ADP-ribosylation site to pertussis toxin due to steric hindrance caused by the receptor.

Both the subunit aggregation state of G_i and G_o and interactions with other membrane components will affect the susceptibility of these G proteins to [32P]ADP-ribosylation (Neer *et al.*, 1984; Van Dop *et al.*, 1984; Panico *et al.*, 1990; van der Ploeg *et al.*, 1992). The carboxyterminal region of the G protein is involved in interactions with receptors (Boege *et al.*, 1991) and may sterically hinder modification of the cysteine that is ADP-ribosylated (West *et al.*, 1985). Because guanyl nucleotides affect the aggregation state and conformation of G proteins, we compared the effects of guanyl nucleotides in modulating pertussis toxin-induced ADP-ribosylation.

GDP augmented the level of ADP-ribosylation produced by pertussis toxin to a greater extent in *S. altivelis* than in *S. alascanus* brain membranes (Fig. 6); only the highest concentration of GDP tested (1000 μM) was effective in *S. alascanus* membranes. GDP may enhance [32P]ADP-ribosylation by promoting the formation and stabilization of the substrate $G_{\alpha\beta\gamma}$ holoprotein (Birnbaumer *et al.*, 1990) and by dissociating the $G_{\alpha\beta\gamma}$ holoprotein from unoccupied receptors (Panico *et al.*, 1990; van der Ploeg *et al.*, 1992). This latter action would facilitate ADP-ribosylation by relieving the steric hindrance caused by receptor coupling to heterotrimeric G proteins (van der Ploeg *et al.*, 1992). Thus, by promoting the dissociation of the unoccupied receptor-$G_{\alpha\beta\gamma}$ complex, GDP may expose the carboxyterminal cysteine residue to pertussis toxin. In *S. alascanus* brain membranes, G proteins more tightly coupled to high-affinity forms of receptors would have a reduced affinity for GDP.

In the presence of GTPγS, the extent of incorporation of the radiolabel into G protein α subunits of *S. alascanus* brain membrane was markedly suppressed; *S. altivelis* G protein α subunits were much less sensitive to this inhibitory modulation (Fig. 7). The results with *S. alascanus* membranes are in accordance with the demonstration that GTPγS markedly inhibits pertussis toxin-catalyzed ADP-ribosylation of a 40 kDa protein in rat glioma membranes (Milligan, 1987). GTPγS promoting the dissociation of G_i and G_o protein heterotrimers into $\alpha \cdot$GTPγS and $\beta\gamma$ subunits may underlie this effect, although a GTPγS induced conformational change in G proteins has also been reported to inhibit ADP-ribosylation (Mattera *et al.*, 1987; Yi *et al.*, 1991).

Tighter association of receptors and G proteins in *S. alascanus* would result in a larger fraction of coupled receptors. This could promote, relative to uncoupled G proteins, the binding of GTPγS to the unoccupied guanyl nucleotide binding site, eliciting the dissociation of the G protein subunits, and a resultant decrease in [32P]ADP-ribosylation. This would be less likely to occur in *S. altivelis* brain membranes inasmuch as the uncoupled population of G protein heterotrimers would be largely GDP-liganded. The modest GDP-enhancement of ADP-

ribosylation in *S. altivelis* membranes could result from the stabilization and recruitment of that fraction of G proteins that are either uncoupled or loosely coupled, and unliganded by GDP.

This reasoning is supported by several observations. First, unoccupied receptors can stimulate, albeit weakly, G protein activation; signaling is a consequence of an agonist-induced increase in the efficacy of G protein-receptor coupling (Birnbaumer *et al.*, 1990; Mukai *et al.*, 1992). Second, the high affinity states of receptors, i.e., G protein-coupled states, can be abolished by the addition of guanyl nucleotides (Mukai *et al.*, 1992). Third, solubilized receptors are often isolated precoupled with G proteins and this coupling can be disrupted by the addition of guanyl nucleotides (Leid *et al.*, 1989).

The coupling of G proteins and receptors is sensitive to the degree of membrane order (Casadó *et al.*, 1992). In porcine brain cortical membranes, increased membrane viscosity abolishes the high affinity (G protein-coupled) A_1 adenosine receptor state (Casadó *et al.*, 1992). In hepatocytes, membrane viscosity influences the lateral movement of components of the glucagon-G protein-adenylyl cyclase complex necessary for signal transduction (Houslay *et al.*, 1980, 1981). The brain membrane phospholipid and fatty acid compositions of the *Sebastolobus* species are identical, and the different hydrostatic pressures experienced by the species will impose different degrees of membrane order (Siebenaller, 1991). A less efficient coupling of receptors in brain membranes of the deeper-living *S. altivelis* may reflect a requirement for greater conformational flexibility and mobility for elements functioning in the more ordered membrane environment of *S. altivelis* at its *in situ* pressure. That the G_i and G_o proteins of *S. altivelis* membranes are better substrates for pertussis toxin-catalyzed [^{32}P]ADP-ribosylation may reflect this greater conformational flexibility. Based on these considerations, we predict that, in addition to the A_1 adenosine receptor, other G protein-coupled receptor signaling complexes will be sensitive to pressure perturbation.

The precise homology of the G protein α subunits in fish membrane to mammalian α subunits cannot be ascertained at present, although comparisons of cloned α subunit genes from a variety of organisms suggest a slow evolution of these genes, as would be expected for proteins with such important biological functions (Yokoyama and Starmer, 1992). The array of G protein subunits identified in three fishes from two families with antisera directed against mammalian consensus peptides supports the evolutionary conservation of these proteins. But the unique protein in fish brain identified with GC/2 antiserum (Fig. 4) and the altered electrophoretic mobilities of $G_{i\alpha3}$ in the *Sebastolobus* species (Fig. 4) suggest that there may be phylogenetic diversity as well. The central role played by G proteins in transmembrane signaling makes them an important site for adaptation to environmental parameters.

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